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In vitro nematicidal effect of *Chenopodium ambrosioides* and *Castela tortuosa n*-hexane extracts against *Haemonchus contortus* (Nematoda) and their anthelmintic effect in gerbils

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Abstract

The *in vitro* nematicidal effect of *Chenopodium ambrosioides* and *Castela tortuosa n*-hexane extracts (E-Cham and E-Cato, respectively) on *Haemonchus contortus* infective larvae (L3) and the anthelmintic effect of these extracts against the pre-adult stage of the parasite in gerbils were evaluated using both individual and combined extracts. The *in vitro* confrontation between larvae and extracts was performed in 24-well micro-titration plates. The results were considered 24 and 72 h post confrontation. The *in vivo* nematicidal effect was examined using gerbils as a study model. The extracts from the two assessed plants were obtained through maceration using *n*-hexane as an organic agent. Gerbils artificially infected with *H. contortus* L3 were treated intraperitoneally with the corresponding extract either individually or in combination. The results showed that the highest individual lethal *in vitro* effect (96.3%) was obtained with the E-Cham extract at 72 h post confrontation at 40 mg/ml, followed by E-Cato (78.9%) at 20 mg/ml after 72 h. The highest combined effect (98.7%) was obtained after 72 h at 40 mg/ml. The *in vivo* assay showed that the individual administration of the E-Cato and E-Cham extracts reduced the parasitic burden in gerbils by 27.1% and 45.8%, respectively. Furthermore, the anthelmintic efficacy increased to 57.3% when both extracts were administered in combination. The results of the present study show an important combined nematicidal effect of the two plant extracts assessed against L3 in gerbils.

Introduction

One of the most pathogenic parasitic nematodes affecting small ruminants worldwide is the species *Haemonchus contortus* (Waller *et al.*, 2006). This is considered to be one of the most severe parasites, causing anaemia, weight loss, hypoproteinemia and even death in young animals (Risso *et al.*, 2015). Haemonchosis and other nematodiases in ruminants cause significant economic losses worldwide. Weight loss due to haemonchosis and other nematodiases has been estimated at 27% of the total production, and milk production losses are estimated to be 29% of the total volume (Qamar *et al.*, 2011).

Nematodiasis in small ruminants has been addressed from different perspectives. The most commonly used practice to control ruminant parasitic nematodes is through chemical anthelmintic drugs. This strategy helps to reduce the parasitic burden in the infected animals; however, the efficacy of these drugs is rapidly diminishing due to the imminent presence of anthelmintic resistance (Peña-Espinoza *et al.*, 2014). A number of alternatives for controlling and preventing nematodiasis, including the use of nutritional improvement, grazing management, copper particles, immunization, and biological control, have been proposed (Torres *et al.*, 2012). In recent decades, the potential use of plants and their extracts as natural de-wormers against ruminant parasitic nematodes has gained attention (Rochfort *et al.*, 2008; Chan-Pérez *et al.*, 2016). Similarly, the use of medicinal plants with nematicidal activity is now considered an important area of research, playing a crucial role in the treatment of parasitic diseases that affect ruminants, specifically sheep and goats, from a sustainable perspective. This alternative is considered a promising tool for controlling parasites while also reducing the harmful effects that occur with the overuse of synthetic chemical anthelmintic

drugs (Squires *et al*., 2010a, b; Mendoza-de Gives *et al*., 2012), i.e. anthelmintic resistance, noxious environmental effects and even public health risks (Beyene, 2016). *Chenopodium ambrosioides* and *Castela tortuosa* are two Mexican plants that are being investigated for medicinal properties. *Chenopodium ambrosioides* is an edible plant commonly consumed by Mexican people as a seasoning in traditional cuisine, and *C. tortuosa* is used in traditional medicine for antidiarrhoeic, anti-amoebiasis and other treatments (Galicia-Aguilar *et al*., 2008). Other important medicinal properties have been identified in these plants in traditional Mexican medicine, such as antioxidant (Mercado-Mercado *et al*., 2013), anticancer (Gawlik-Dziki *et al*., 2013) and anti-parasitic activities (Jabbar *et al*., 2007; Anderson, 2014). Clean technologies for livestock and agriculture production require the use of traditional alternatives to control a number of diseases. The use of plants or plant extracts with anti-parasitic effects to control animal parasitic nematodes has gained interest among researchers worldwide during the last few decades (Ugwuoke *et al*., 2011). The present study was designed to (1) assess the *in vitro* nematicidal effect of *C. ambrosioides* and *C. tortuosa* *n*-hexane extracts (E-Cham and E-Cato, respectively) against *H. contortus* infective larvae (L3), and (2) evaluate the *in vivo* anthelmintic effect with either a single or combined use of the extracts in gerbils as a study model.

Materials and methods

Location

This research was conducted at the Laboratory of Helminthology at the National Centre for Disciplinary Research in Veterinary Parasitology (CENID-PAVET) of the National Institute of Agricultural, Forestry and Livestock Research (INIFAP) in Jiutepec Municipality, Morelos State, Mexico. Plant extractions were performed at CIByC-UAEM in Cuernavaca Municipality, Morelos, Mexico.

Plant material

Both *C. ambrosioides* and *C. tortuosa* plants were acquired at a local market in the town centre of Cuernavaca City, Morelos, Mexico. Dr Gabriel Flores, an expert in botanical taxonomy from the Autonomous University of the State of Morelos, Mexico, identified the two plants. One sample of each plant was deposited in the Herbarium of the Research Centre in Biodiversity and Conservation at the same institution. The key registration numbers of the plants are *C. ambrosioides* (HUMO 2900) and *C. tortuosa* (HUMO 7019).

Plant extracts

The plant material was processed through organic maceration (Azwanida, 2015; Ngaha-Njila *et al*., 2017). The aerial parts of the fresh plants, including the leaves and stems, were dried in a closed chamber with electrical heating at 52°C for two days. Once the plants were completely dried, 1 kg of each dried plant was placed in a 10-l glass container, and 5 l of *n*-hexane was added to completely cover the plant with the organic solvent. The plant material remained under these conditions for 3 days, and the process was repeated two consecutive times. The solvent was removed by distillation under reduced pressure with a rotary evaporator (BUCHI mark 205). The dried extracts were compared by thin layer chromatography (TLC). The samples were gathered based on their similar chemical patterns and were subsequently mixed, generating a 2.22% total yield. Different concentrations of these extracts, 0.6, 1.25, 2.50, 5, 10, 20 and 40 mg/ml, were prepared with 3% Tween 20 in distilled water (Palacios-Landín *et al*., 2015). The final extracts were referred to as E-Cham (from *C. ambrosioides*) and E-Cato (from *C. tortuosa*).

Experimental animals

A population of male and female gerbils 3 months of age, weighing 35 g, were obtained from a commercial pet store. The gerbils were de-wormed with Albendazole (Valbazen) administered orally at 50 µl per day for 4 days. In a preliminary test, the optimal oral dose of L3 to infect gerbils was determined to be 20,000 L3 (exsheathed larvae) to establish a good infection. Similarly, the optimal date after infection to sacrifice gerbils to evaluate the potential reduction in parasitic burden was 12–13 days (unpublished data).

Parasitological techniques

Production of H. contortus L3

A 3-month-old male hair sheep, weighing 35 kg, was artificially infected with 350 (L3) per kg of body weight. Faecal samples were collected directly from the rectum of the sheep after the 20-day pre-patent period. The faecal samples were processed through a faecal culture technique in plastic bowls to obtain as many infective larvae as possible (Liébano-Hernández, 1998). The faecal cultures were incubated for 6 days at room temperature (18–25°C) to obtain infective larvae. The larvae were recovered from faecal cultures using the Baermann funnel technique (OEPP/EPPO Bulletin, 2013). The recovered larvae were filtered through lens paper using the Baermann technique. The larvae were washed via gravity density centrifugation with a 40% sucrose solution at 3500 rpm for 5 minutes; a pellet formed in the white ring interphase corresponding to the larvae. The larvae were recovered and re-suspended in sterile distilled water to eliminate sucrose residues (Figueroa Castillo *et al*., 2015). The L3 were exsheathed using 0.18% sodium hypochlorite for 6–8 minutes at room temperature (18–25°C); the larvae were then rinsed and subsequently re-suspended in sterile distilled water (Ramírez *et al*., 2006). Counting of larvae was performed using five 10-µl aliquot drops, and the total number of larvae present in a known volume was estimated.

In vitro *bioassays to determine the 50% and 90% lethal concentrations against infective larvae of* H. contortus

Different concentrations of E-Cham, E-Cato and the combination of both extracts (0.6, 1.25, 2.50, 5, 10, 20 and 40 mg/ml) were evaluated to determine the 50% (LC_{50}) and 90% (LC_{90}) lethal concentrations (López *et al*., 2015). Each concentration was assessed considering three replicates. A concentrated solution, 'stock solution' non-diluted extract (40 mg/ml), was used initially; fold dilutions were prepared from this concentration. The test was conducted in 24-well cell culture plates (Nunc, InterMed, Denmark), with 500 µl of each corresponding extract deposited into each well of the three replicates. Tween 20 (3%) and distilled water were used as controls, as was fenbendazole. Subsequently, 500 exsheathed L3 were added to each well of the different treatments and incubated at room temperature (18–25°C). The results were considered by counting the number of live and dead larvae

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in ten 5-µl drops at 24 and 72 h post confrontation, as previously described (López-Arellano *et al.*, 2006).

In vivo *bioassays to assess the nematicidal effect of plant extracts against* H. contortus *in gerbils*

Thirty gerbils (previously described) were used. All animals were treated with dexamethasone (Azium, Schering Plough Laboratories) using an intra-muscular route at a total dose of 100 µl per day for 2 consecutive days prior to artificial infection to improve L3 establishment. Every animal was infected orally with 3 ml of an aqueous suspension containing 20,000 exsheathed L3. At 9 days post infection, six groups of five gerbils each were assigned randomly as follows: groups 1 and 2 were treated intraperitoneally (IP) with E-Cham and E-Cato extracts, respectively, at 40 mg/kg BW. Group 3 comprised gerbils treated with a combination of extracts in a 1 : 1 relationship at the same dose and route of administration. Group 4 comprised gerbils IP treated with 100 µl of 3% Tween 20; this was considered a negative control group. Group 5 comprised animals treated with only water (100 µl) as another negative control (placebo) to indicate larval viability. Finally, group 6 included animals IP treated with fenbendazole (Sigma-Aldrich) at a unique dose of 10 mg/kg BW (Villar *et al.*, 2007) as a positive control. All animals were sacrificed by cervical dislocation at 13 days post infection, according to the Official Mexican Norm 062-ZOO 1999 regarding technical specifications for the production, care and use of laboratory animals. The animals were necropsied, and the total parasites were recovered from their stomachs. The recovered parasites were eventually counted, and the mean numbers per group were estimated for comparison. Similarly, the gastric mucosa of each animal was placed in artificial gastric juice to simulate stomach digestion to obtain larvae in hypobiosis from mucosa (Campos & Bautista, 1989). Two evaluation criteria were considered: the mean number of nematodes recovered from the treated group with respect to the mean number of nematodes recovered from group 4 (negative control 3% Tween 20), and the mean number of nematodes recovered compared with the mean number of nematodes recovered from group 5 (control: water). In both cases, the mean number of nematodes recovered in the control groups was considered as 100% larval viability.

The reduction percentage was estimated based on the following formula (Mendoza-de Gives *et al.*, 1998):

$$\text{Percentage reduction of parasites} = (\bar{x}_{\text{control}} - \bar{x}_{\text{treatment}} / \bar{x}_{\text{control}}) \times 100$$

where

$\bar{x}_{\text{control}}$ = mean number of nematodes recovered from the negative control groups

$\bar{x}_{\text{treatment}}$ = mean number of nematodes recovered from treated group

Preparation of plant extracts

The aerial parts of fresh plants, including the leaves and stems, were dried in a closed chamber with electrical heating at 52°C for 2 days. Once the plants were completely dried, 1 kg of dried individual plant material was placed into a 10-l glass container, and 5 l of *n*-hexane was added, with the organic solvent completely covering the plant. The plant materials were maintained under these conditions for 3 days, and the process was repeated two consecutive times. The solvent was removed by distillation under reduced pressure with a rotary evaporator, BUCHI mark 205 (von Son de Fernex *et al.*, 2016). The dried extracts were compared by thin layer chromatography (TLC). The plant yields were estimated as follows: (E-Cham = 2.22%; E-Cato = 2.5%). Different concentrations of these extracts, 0.6, 1.25, 2.50, 5, 10, 20 and 40 mg/ml, were prepared with 3% Tween 20 in distilled water (Palacios-Landín *et al.*, 2015).

Statistical analysis

The 50% and 90% lethal concentrations (LC_{50} and LC_{90}) were determined using the Polo statistical programme (LeOra Software). In the *in vivo* assay, the dependent variable was calculated as the mean number of recovered nematodes at necropsy. A comparison among the means of the different groups was conducted through analysis of variance (ANOVA). A complementary Tukey's test was also conducted to determine the difference between treatments, using SAS statistical software version 2002 (Zar, 1999).

Results

In vitro *lethal concentration (LC_{50}) and (LC_{90}) and nematicidal efficacy percentages against* H. contortus *L3*

The 50% and 90% *in vitro* lethal concentrations against L3 attributed to the effect of E-Cham and E-Cato extracts used either individually or combined at 24 and 72 h post confrontation are shown in table 1. Concentration-dependent lethal activity was identified in most of the treatments, reaching the maximum activity at 40 mg/ml either at 24 or 72 h post confrontation. Notably, the Polo statistical programme predicted 90% LC at concentrations higher than 40 mg/ml. The highest lethal effect against L3 was observed when E-Cham was used individually. An increase in the lethal activity of both combined extracts was identified after a 72-h interaction at 20 and 40 mg/ml concentrations.

Apart from this finding, no significant increase in the *in vitro* lethal activity was identified when the two extracts were used in a combined manner at all assessed concentrations. The highest *in vitro* lethal activity (close to 100%) was obtained with the combined use of both extracts at 72 h post confrontation at 40 mg/ml concentrations.

In vivo *nematicidal activity of plant extracts in gerbils*

The *in vivo* nematicidal activity of E-Cham and E-Cato extracts either individually or combined at different concentrations in gerbils is shown in table 2. When administered individually in gerbils, the E-Cato and E-Cham extracts reduced the parasitic burden by 27.1% and 45.8%, respectively, when compared with the control group in water. Moreover, the anthelmintic efficacy increased to 57.3% ($P > 0.05$) when both plant extracts were used as a combined inoculum.

Discussion

The results of the present study provide evidence that the organic extracts E-Cham and E-Cato from *C. ambrosioides* and *C. tortuosa,* respectively, possess an *in vitro* nematicidal effect when used individually. A clear synergistic effect was also recorded when the two extracts were used at the two highest concentrations after a 72-h interaction. The combination of other plant/plant extracts has demonstrated better results than using individual plant materials alone. For example, better results were obtained with the combined use of methanolic extracts obtained from *Caesalpinia crista* and *Chenopodium album* flowers than when the extracts were used individually against the larvae and eggs of trichostrongyle nematodes (Jabbar *et al.*, 2007). However, the

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Table 1. Individual and combined *in vitro* nematicidal activity of *Chenopodium ambrosioides* and *Castela tortuosa* *n*-hexane extracts (E-Cham and E-Cato, respectively) against *H. contortus* L3, expressed as % efficacy, with 50% and 90% lethal concentrations after 24 and 72 h.

	24 h after confrontation			72 h after confrontation		
Concentration (mg/ml)	E-Cham*	E-Cato**	E-Cham/E-Cato***	E-Cham*	E-Cato**	E-Cham/E-Cato***
40	91.1 (±4.7) ab	53.8 (±23.4)	78.5 (±5.2) a	96.3 (±1.2) a	76 (±5.5) a	98.7 (±56.6) ab
20	92.8 (±6.6) a	43.9 (±17.2)	47.2 (±34.2) a	89.3 (±6.0) ab	78.9 (±4.4) a	92.2 (±3.7) a
10	77.5 (±3.8) ab	17.2 (±16.4)	66 (±13.2) ab	93.4 (±2.8) a	48.7 (±24.2) ab	80.1 (±16.2) ab
5	58.2 (±9.6) bc	14.8 (±16.4)	42.2 (±15.7) ab	83.9 (±11.0) ab	29.8 (±30.0) ab	25 (±5.0) ab
2.5	7.6 (±7.2) cd	6.6 (±0.3)	8.2 (±7.2) ab	67 (±5.2) abc	22.3 (±28.9) ab	19 (±23.2) ab
1.25	8.2 (±11.5) d	8.9 (±19.5)	15.8 (±34.8) ab	47.9 (±6.5) bc	7.4 (±12.4) b	28 (±12.7) ab
0.6	0 (±8.8) d	7.2 (±16.7)	0 b	26 (±26.5) c	4.0 (±4.0) ab	13.6 (±0.23) b
LC_{50}	6.3	26	10.7	1.5	17.3	6.5
LC_{90}	28.4	98.2	50.2	16.7	64.2	21.4

24 h: *$P < 0.0001$; CV = 19.8 ** $P = 0.3236$; CV = 85.1 *** $P < 0.0267$; CV = 48.4
72 h: * $P < 0.0002$; CV = 17.4 ** $P < 0.0171$; CV = 30.4 *** $P < 0.0467$; CV = 60
Means with different letters are significantly different according to Tukey's test.

activity of both organic extracts was dose dependent, because the *in vitro* nematicidal effect increased proportionally with increasing plant concentration. The *in vitro* dose effect has been reported previously for other plant extracts. For example, the larval migration inhibition (LMI) test showed a dose-dependent anthelmintic effect for *Acacia pennatula*, *Lysiloma latisiliquum* and *Leucaena leucocephala* extracts (Alonso *et al.*, 2008). In a review of the available literature, the authors of the present study found only one reference describing the results of *C. ambrosioides* and *C. tortuosa* *in vitro* nematicidal activity of an *n*-hexane extract of these plants. The results in this reference showed that *C. ambrosioides* possesses a high *in vitro* nematicidal effect (99.7%) against *H. contortus* fourth instar larvae after a 96-h interaction at the same concentration shown in the present study (Galicia-Aguilar *et al.*, 2008). These authors found that the *n*-hexane extract of *C. tortuosa* achieved the highest *in vitro* nematicidal effect (of 85.8%) after a 96-h interaction at the same larval stage. These results are similar to those obtained in the present study using L3 stage larvae. Furthermore, the *in vitro* nematicidal effect using a combination of both extracts was higher than the individual effects of either plant extract after a 72-h confrontation. However, in the present study, for both *in vitro* and *in vivo* assays, we used two control groups: L3 larvae in a Tween 20 suspension and L3 larvae contained in only water. Tween 20 was used because it was not possible to dissolve the extracts in water due to the immiscibility of these compounds. Tween 20 has been used in similar research studies as a suitable plant extract dissolvent because most L3 are typically not affected either *in vitro* or *in vivo* (De Jesús Gabino *et al.*, 2010). However, the results of the present study show that this dissolvent had certain lethal effects against nematodes, which were considered when we estimated the efficiency of the extracts.

The anthelmintic effectiveness reported in the present study could likely be improved by increasing the dose, using other routes of administration, assessing different vehicles to increase the bioavailability of bioactive compounds, or perhaps obtaining plant extracts using other organic solvents and purifying the active compounds in these extracts.

This work contributes to the scientific knowledge on the nematicidal activity of *n*-hexane extracts from medicinal plants against *H. contortus* using gerbils as an *in vivo* model of study. The plant extracts assessed in the present study prompted a phytochemical analysis to elucidate the compounds responsible for the consideration of nematicidal activity as viable and sustainable potential

Table 2. Individual and combined nematicidal activity of *C. ambrosioides* and *C. tortuosa* *n*-hexane extracts (E-Cham and E-Cato, respectively) against *H. contortus* in artificially infected gerbils, expressed as the percentage reduction of *H. contortus* larvae.

Treatment	Total recovered larvae	Larvae recovered from gerbil stomachs	*	**
E-Cham	353	70.6 (±41.04) a	45.86%	32.2% a
E-Cato	475	95 (±28.22) a	27.15%	8.6% a
E-Cham/E-Cato	278	55.6 (±24.29) b	57.36%	46.5% b
Tween 20, 3% (Control)	520	104 (±41.23) a	20.24%	–
Water (Control)	652	130.4 (±51.53) a	–	–
Fenbendazole	11	3 (±2.22) c	97.7%	97.1%

Different letters indicate significant differences between treatments.
Dose of *n*-extracts of both plants was 40 mg/kg BW; ANOVA: $P < 0.0005$; CV = 45.5.
* Percentage of population reduction considering the water control group as 100%.
** Percentage reduction of the population in the control group prepared with Tween 20 as 100%.

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tools for controlling nematodiasis in small ruminants and other species of economic impact in the livestock industry. The present study is the first to establish a synergistic anthelmintic effect of two *n*-hexane plant extracts as a potential de-wormer administered in gerbils against nematodes, and this effect can be considered a foundation for future research focused on a potential natural de-wormer in sheep and other economically important animal species. The purification of these extracts, followed by the elucidation of the bioactive compounds responsible for the anthelmintic effect, is currently in preparation for publication by our research group. Exploring the potential use of these plant extracts in sheep as a potential de-wormer implies the performance of some important steps, including obtaining and identifying the molecules responsible for the anthelmintic activity and establishing the concentration of the active compound to be administered to sheep, which depends on the pharmacokinetic (PK) and pharmacodynamic (PD) properties of the molecules once administered to the animals. PK/PD modelling can be used to identify the relationship based on the plasma concentration profile to eventually estimate a proper dosage regime (Toutain & Lees, 2004). Other alternative strategies for using these plants as natural de-wormers could be explored; for example, identifying the content of bioactive compounds in the foliar system of these plants as well as their palatability in sheep and lack of toxicity could be important to assess a potential beneficial effect of the consumption of these plants in reducing the parasitic burden in sheep flocks maintained under grazing conditions.

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Conflict of interest. None.

Ethical standards. The sheep were strictly maintained under the Norma Oficial Mexicana (Official Rule Number) NOM-051-ZOO-1995 (http://www.senasica.gob.mx) and the LEY Federal de Sanidad Animal (Federal law for animal health) DOF 07-06-2012 (http://diputados.gob.mx/LeyesBibliop/ref/lfsa.htm). These guidelines specify that all procedures performed in studies involving animals must follow the Federal Law and Official Rule strictly in accordance with the ethical standards of INIFAP. Furthermore, the guidelines are based, in part, on the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animals Resources Commission on Life Sciences, National Research Council, 1996.

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